FEBS 29946 FEBS Letters 579 (2005) 5241–5246

Adenovirus-mediated silencing of Synaptotagmin 9 inhibits Ca^{2+}-dependent insulin secretion in islets

Mariella Iezzi[a], Lena Eliasson[b], Mitsunori Fukuda[c], Claes B. Wollheim[a,*]

[a] Department of Cell Physiology and Metabolism, University Medical Center, 1211 Geneva 4, Switzerland
[b] Department of Experimental Medical Science, Division of Diabetes, Metabolism and Endocrinology, BMC B11, 221-84 Lund, Sweden
[c] Fukuda Initiative Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Received 15 July 2005; revised 18 August 2005; accepted 21 August 2005

Available online 6 September 2005

Edited by Robert Barouki

Abstract **Synaptotagmins (Syts) are involved in Ca^{2+}-dependent insulin release. However, which Syt isoform is functional in primary β-cells remains unknown. We demonstrate by electron microscopy of pancreatic islets, the association of Syt 9 with insulin granules. Silencing of Syt 9 by RNA interference adenovirus in islet cells had no effect on the expression of Syt 5, Syt 7 and Syt 3 isoforms. The latter was localized at the plasma membrane of pancreatic polypeptide cells. Insulin release in response to glucose or tolbutamide was strongly inhibited in Syt 9 deficient islets, whereas exocytosis potentiated by raising cAMP levels, was unaltered. Thus, Syt 9 may act as Ca^{2+} sensor for β-cell secretion.**

Keywords: Pancreatic islets; Insulin exocytosis; Electron microscopy; RNA interference; β-cell

1. Introduction

Glucose and other insulin secretagogues depolarize the β-cell plasma membrane. The subsequent Ca^{2+} entry triggers release of insulin granules [1]. Among the Ca^{2+}-binding proteins that are potential Ca^{2+} sensors for exocytosis, Synaptotagmins (Syts) are currently the best candidates [2].

At present 15 different mammalian isoforms have been described [2,3]. The abundant synaptic vesicles isoform Syt 1, functions as the major Ca^{2+} sensor for neurotransmitter release [4]. Syt 1 is also present on secretory vesicles in endocrine cells (e.g., chromaffin cells, PC12 cells and pancreatic β-cell lines) and is involved in Ca^{2+}-dependent exocytosis, suggesting a role for Syt 1 as an endocrine Ca^{2+} sensor [5–7]. In PC12 cells, the importance of this isoform has, however, been questioned [8]. Pancreatic β-cells do not express Syt 1 or the highly homologous Syt 2 [7], prompting the question which isoform is predominant in primary cells.

Additional Syt isoforms were proposed to function as Ca^{2+} sensors in neuroendocrine cells including PC12 and insulin-secreting cells [3,9–12]. Consistent with this notion, Syts 3, 5, 7 and 8 were suggested to regulate Ca^{2+}-stimulated release in primary β-cells by introduction of antibody or peptides corresponding to the C_2 domains [9,11]. However, the functional assays used, do not directly prove which Syt isoform is the candidate Ca^{2+} sensor. Moreover, Syt 5 was only detected in islet α-cells [12,13], Syt 8 is an atypical isoform lacking Ca^{2+} binding sites [2], whereas the cellular localization of Syt 3 is still controversial [9–11]. Finally, Syt 7 immunoreactivity was detected in insulin-containing islet cells [10] but its precise subcellular distribution in the native β-cell remains to be determined.

Our previous immunofluorescence study on isolated β-cells revealed that Syt 9, closely related to Syt 1 [14], is associated with insulin granules and controls Ca^{2+}-dependent exocytosis in the cell line INS-1E [12]. Here, we investigate the Syt 9 localization at the ultrastructural level and determine whether this isoform functions as Ca^{2+} sensor for insulin secretion in the β-cell.

2. Materials and methods

2.1. Materials

The mouse monoclonal antibody against rat Syt 9 was purchased from BD Biosciences (Basel, Switzerland). Anti-Syt 3 and anti-Syt 7-N rabbit polyclonal antibodies were prepared as previously described [13,15]. The monoclonal antibodies specific for syntaxin, insulin and glucagon were from Sigma (Buchs, Switzerland). Anti-pancreatic polypeptide rabbit polyclonal antibody was from Peninsula Laboratories (Belmont, CA, USA). Anti-somatostatin rat monoclonal antibody was from Biogenesis (Poole, UK). The guinea pig antibody against insulin was kindly provided by Dr. P. Meda (University of Geneva, Switzerland). Tolbutamide, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma.

2.2. Cell culture

The rat clonal β-cell line INS-1E derived from parental INS-1 cells was cultured in RPMI 1640 medium and other additions as described [16]. Pancreatic islets were isolated from male Wister rats (200–300 g) by collagenase (Serva Electrophoresis, Heidelberg, Germany) digestion and handpicking. Islets were then cultured free floating in RPMI 1640 medium supplemented as for INS-1E cells, for 24 h before adenovirus infection.

2.3. Preparation of Syt 9 silencing adenovirus and infection

The Syt 9 hairpin small interfering RNA (siRNA) template containing the U6-RNA promoter was constructed as described [12]. The siRNA insert was cloned into the pAxcw adenoviral backbone and the recombinant adenovirus (AdsiRNASyt9) was generated according to manufacturer's instructions (Takara Shuzo, Biomedical Group, Otsu, Shiga, Japan). AdCALacZ express bacterial β-galactosidase and was used as a control adenovirus.

*Corresponding author. Fax: +41 22 379 55 43.
E-mail address: claes.wollheim@medecine.unige.ch (C.B. Wollheim).

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; Syt, Synaptotagmin

0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.047

INS-1E cells or isolated islets were infected for ~20 h at 50 virus particles/cell or at 3×10^5 virus particles/islet (100 virus particles/cell, assuming 3000 cells/islet), respectively, washed once and further cultured during 3 days before experiments.

2.4. Immunofluorescence microscopy

Infected INS-1E cells and trypsin-dispersed islet cells [17], were seeded on glass cover slips, fixed for 10 min in 4% paraformaldehyde and permeabilized for 1 h in PBS containing 0.1% saponin and 0.5% BSA. The cells were incubated with primary antibodies overnight at 4 °C and then exposed to FITC- or rhodamine-conjugated secondary antibodies for 1 h at room temperature. The double labelling with anti-pancreatic polypeptide and anti-Syt 3 antibodies was performed with the Tyramide Signal Amplification Kit (Molecular Probes, Basel, Switzerland). Samples were analysed using a Zeiss laser confocal microscope (LSM 510, Zurich, Switzerland).

2.5. Western blotting

The homogenates from infected islets were analysed by Western blotting as described [12].

2.6. Electron microscopy

Intact rat islets were fixed in 1% paraformaldehyde and 2.5% glutaraldehyde for 1 h at 4 °C. The islets were then embedded in type VII agarose (Sigma), cryoprotected in glycerol and embedded in Lowecryl HM20 by cryofixation and freeze-substitution. Ultrathin sections (60–80 nm) were labelled with the mouse anti-Syt 9 antibody and a goat anti-mouse secondary antibody conjugated to 10 nm gold particles (Amersham Biosciences, Uppsala, Sweden). The sections were examined using a JEOL 1230 electron microscope (JEOL-USA, Inc. Peabody, MA, USA). Gold particles within an area of 30 nm outside/inside the membrane of secretory granules were counted as being at the granular membrane. Indeed, gold particles can be situated within this distance from their respective epitopes, reflecting the size of the interposed immunoglobulins [18]. The density of the gold particles was calculated using software package (Scion Corporation, ML, USA).

2.7. Insulin secretion

Infected islets (10 islets/tube) were preincubated for 60 min in Krebs-Ringer-Bicarbonate buffer (KRB) [16] containing 2.8 mM glucose and then incubated during 30 min in KRB supplemented with stimulators as indicated. Insulin content was determined from acid-ethanol extracts. Insulin was measured by radioimmunoassay.

3. Results and discussion

3.1. Intracellular localization of Syt 9

To elucidate the subcellular distribution of Syt 9 in rat pancreatic islets, we performed immuno electron microscopy. The micrographs in Fig. 1 show that Syt 9 is specifically localized at the membrane of insulin secretory granules in β-cells, albeit at low density. It was calculated that 88% of the insulin granule labelling is concentrated to the limiting membrane. The immunogold density in the secretory granules compared to the remainder of the cell is 1.49 ± 0.24 and 0.11 ± 0.02 particles/μm^2, respectively ($n = 32$ sections of an equal number of cells; 6 islets isolated from 2 rats; $P < 0.001$). No staining is observed at the plasma membrane or in the mitochondria. Moreover, the density of the gold particles in the granules is not changed even when the antibody is concentrated 10-fold (compare Fig. 1 panel C to A and B).

3.2. Repression of Syt 9 in islet cells

We have demonstrated that Syt 9 is involved in Ca^{2+}-evoked insulin exocytosis from INS-1E cells [12]. To address the question if Syt 9 constitutes the Ca^{2+} sensitive protein

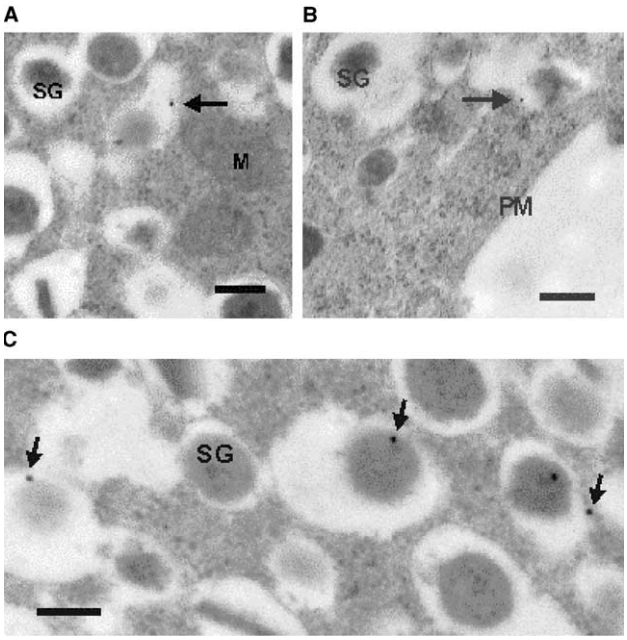

Fig. 1. Immuno electron microscopic localization of Syt 9 on ultrathin cryosections of pancreatic β-cells. The gold particles appear as black dots. Immuno gold labelling at the membrane of secretory granules is indicated by arrows. Anti-Syt 9 antibody was diluted 1/100 (panels A and B) and 1/10 (panel C). Secretory granules (SG), mitochondria (M), plasma membrane (PM). Bar 200 nm.

for secretion in primary β-cells, we generated an adenovirus directing the synthesis of siRNA targeted against Syt 9 (AdsiRNASyt9).

As shown by immunofluorescence, infection of AdsiRNASyt9 in INS-1E cells, used as control cells, potently diminishes the level of endogenous Syt 9 (Fig. 2A). A dramatic decrease of Syt 9 expression level (>80%) is also detected in homogenates of infected islets (Fig. 2B). Considering that under our experimental conditions, 80–90% of islet cells are efficiently infected (as determined by GFP fluorescence, see also [17]), this indicates that Syt 9 is strongly reduced in siRNA-containing islet cells. Indeed, as revealed in dispersed islet β-cells identified by insulin staining, AdsiRNASyt9 almost completely eliminates Syt 9 (Fig. 2C). The silencer effect is specific because the amounts of other endogenous proteins including Syt 5, Syt 7 and syntaxin remain unaffected (Fig. 2B).

3.3. Syt 3 expression in endocrine cells

As the cellular localization of Syt 3 remains unclear [9–11], we analysed the presence of this isoform in pancreatic endocrine cells by confocal microscopy. Double labelling experiments reveal that Syt 3 is not detected in insulin-, glucagon- or somatostatin-containing cells, but is distributed preferentially near the plasma membrane of pancreatic polypeptide cells (Fig. 3A). Similar to Syt 5 and Syt 7, the endogenous expression of Syt 3 is not altered in AdsiRNASyt9-infected islet cells (Fig. 3B).

Syt 3 was not found in β-cells but targeted to the plasma membrane when transiently expressed [11] contrasting with previous reports showing the association of this isoform with insulin granules in β-cells [9,10]. Overexpressed Syt 3 in

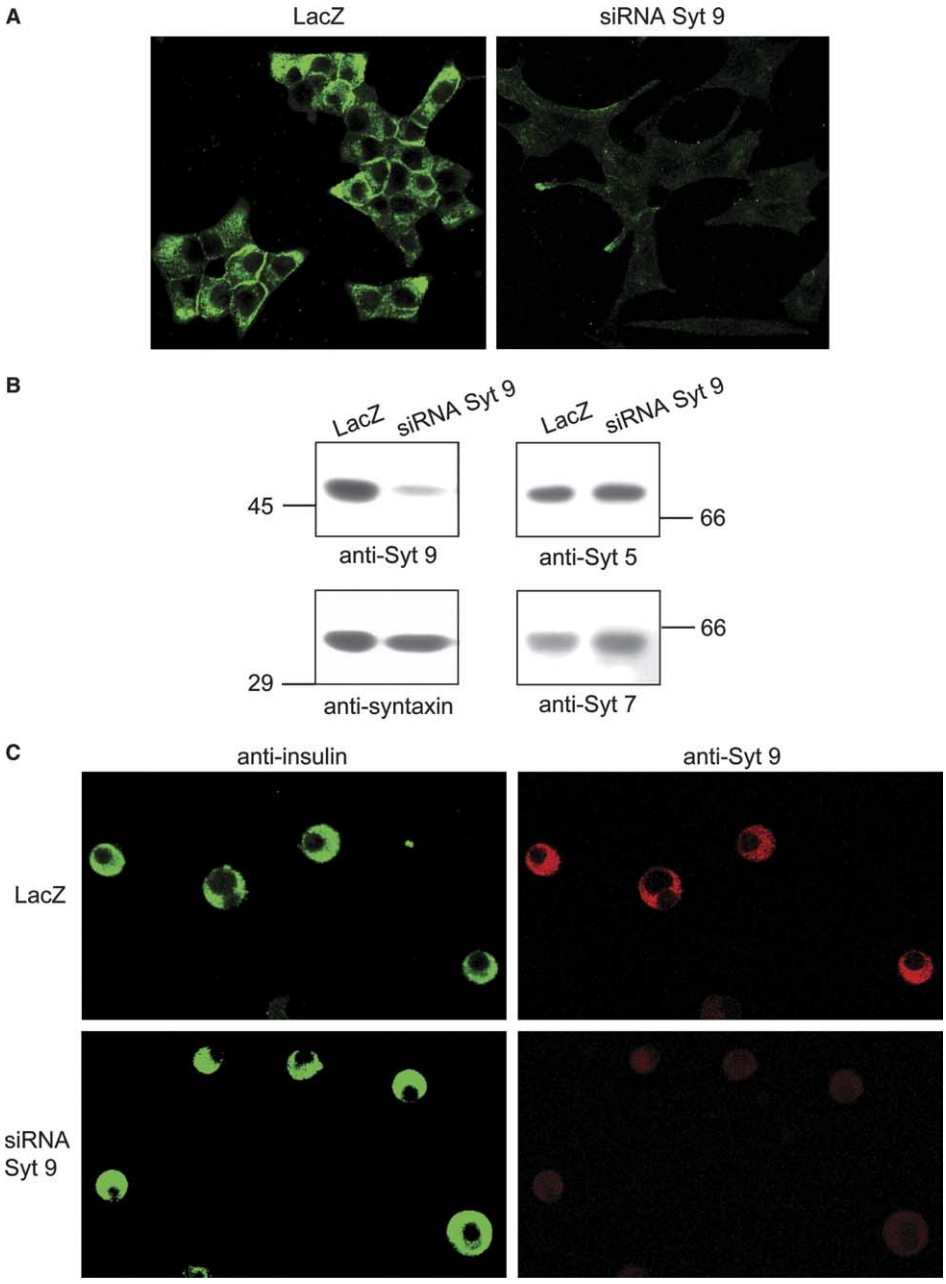

Fig. 2. Adenovirus RNA interference (RNAi)-mediated suppression of Syt 9 in insulin-secreting cells. (A) INS-1E cells infected with the control AdCALacZ (LacZ) or AdsiRNASyt9 (siRNA Syt 9) adenovirus were analysed by confocal microscopy after labelling with the anti-Syt 9 antibody (revealed by FITC-conjugated antibody). (B) Islets were infected with LacZ or siRNA Syt 9, homogenized and equal amounts of protein were analysed by Western blotting using anti-Syt 5, anti-Syt 7, anti-Syt 9 and anti-syntaxin antibodies. The molecular weight markers (kDa) are also shown. (C) Islets infected with LacZ or siRNA Syt 9 were dispersed and double stained with anti-insulin (green) and with anti-Syt 9 (red).

HIT-T15 or PC12 cells was also located on the plasma membrane [11,13]. Similarly, in nerve terminals endogenous Syt 3 was concentrated on the synaptic membrane and not on synaptic vesicles [20]. Therefore, Syt 3 seems in general to be present at the plasma membrane where it may form heterodimers with other Syt isoforms [21].

3.4. Role of Syt 9 in insulin secretion

We next assessed the impact of Syt 9 suppression in islet insulin exocytosis. As depicted in Fig. 4A, basal insulin release is not significantly affected whereas glucose-induced secretion is inhibited by 69% compared to control. The residual release (31%) probably reflects (i) the escape from β-cell adenoviral infection, (ii) the incomplete suppression of endogenous Syt 9 by siRNAs (see Fig. 2C) and (iii) the compensatory effect of Syt 7 isoform also expressed in β-cells [10,19]. To verify whether secretion mediated by another Ca^{2+}-raising agent was diminished, we used the K_{ATP} channel-blocker tolbutamide. Exocytosis stimulated by tolbutamide is also strongly reduced reaching 61% (Fig. 4B). However, the effect of glucose is completely restored in the presence of forskolin and IBMX (Fig. 4C) making a

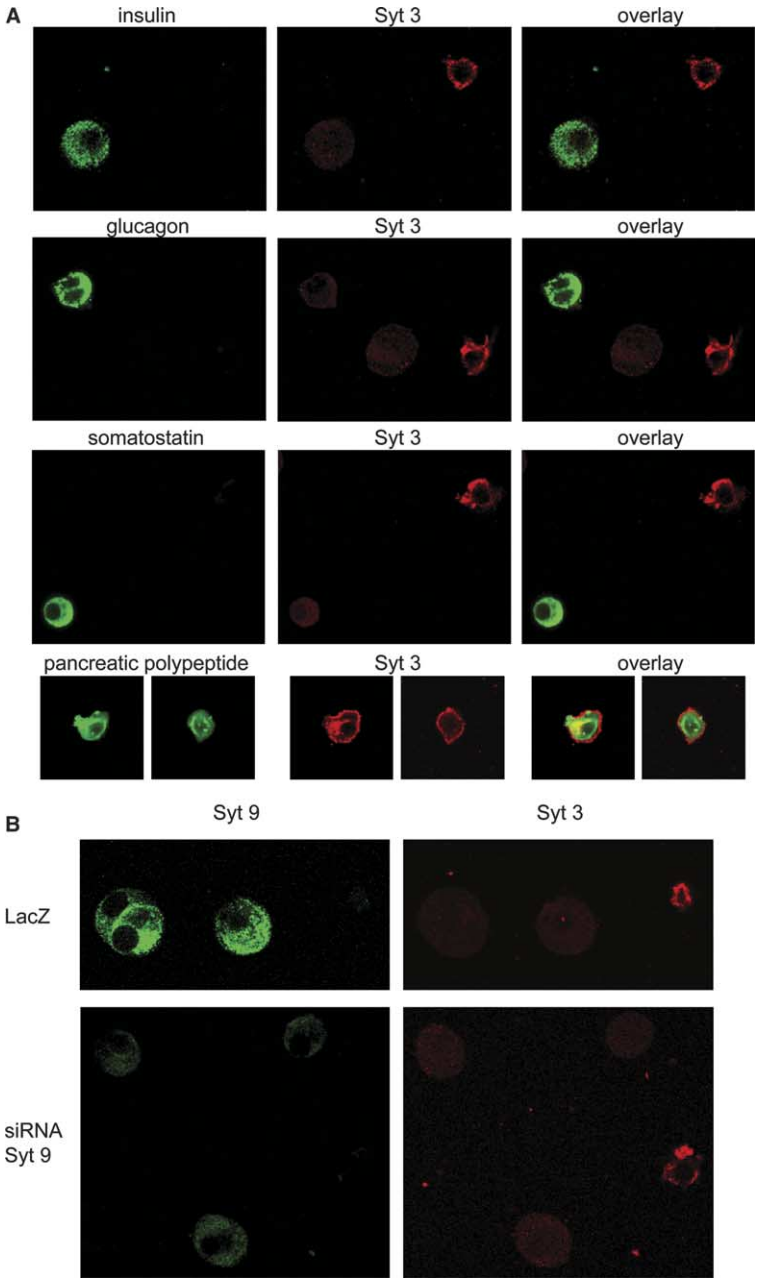

Fig. 3. Syt 3 localization in islet cells and preserved expression after silencing of Syt 9. (A) Pancreatic endocrine cells were analysed by confocal microscopy after double labelling with an antibody against insulin, glucagon, somatostatin or pancreatic polypeptide (green) and with the anti-Syt 3 antibody (red). The right panels show the overlays of the corresponding green and red channels. (B) Islets infected with LacZ or siRNA Syt 9 adenovirus were dispersed and double stained with anti-Syt 9 (green) and with anti-Syt 3 (red).

non-specific effect of the virus on the release machinery unlikely. This also demonstrates that Syt 9 is not required for the process of exocytosis *per se.* In all these experiments Syt 9 silencing does not modify cellular insulin content (not shown). Our data emphasize the importance of Syt 9 for regulation of Ca^{2+}-mediated β-cell secretion and are consistent with the proposed role in dense core vesicle release in PC12 cells [8].

Ca^{2+}-dependent neurotransmission consists of two components: a major fast component insensitive to the Ca^{2+} substitute ion Sr^{2+}, and a slow one potentiated by Sr^{2+} [22]. There is evidence for Syt 9 implication in the slow vesicular release in PC12 cells [8,23]. Regarding the β-cell, insulin secretion has a fast component comprising granules close to Ca^{2+}-channels and a major slow release, with Ca^{2+} affinities of 17 and 1.4 μM, respectively [24]. Sr^{2+} can also support insulin exocytosis albeit with a lower affinity than Ca^{2+} [25,26]. The requirement of Syt 9 for Ca^{2+} and Sr^{2+} in phospholipid binding [22] would therefore be compatible with a role for Syt 9 in regulated secretion. Recently, the heterogeneity of the Ca^{2+} sensitivity of release was further investigated. There is thus a slowly releasable highly calcium-sensitive pool (HCSP) of granules and a less sensitive readily releasable pool [27]. The Ca^{2+}-dependent secretion of the HCSP needs a global concentration of <10 μM [28]. Since

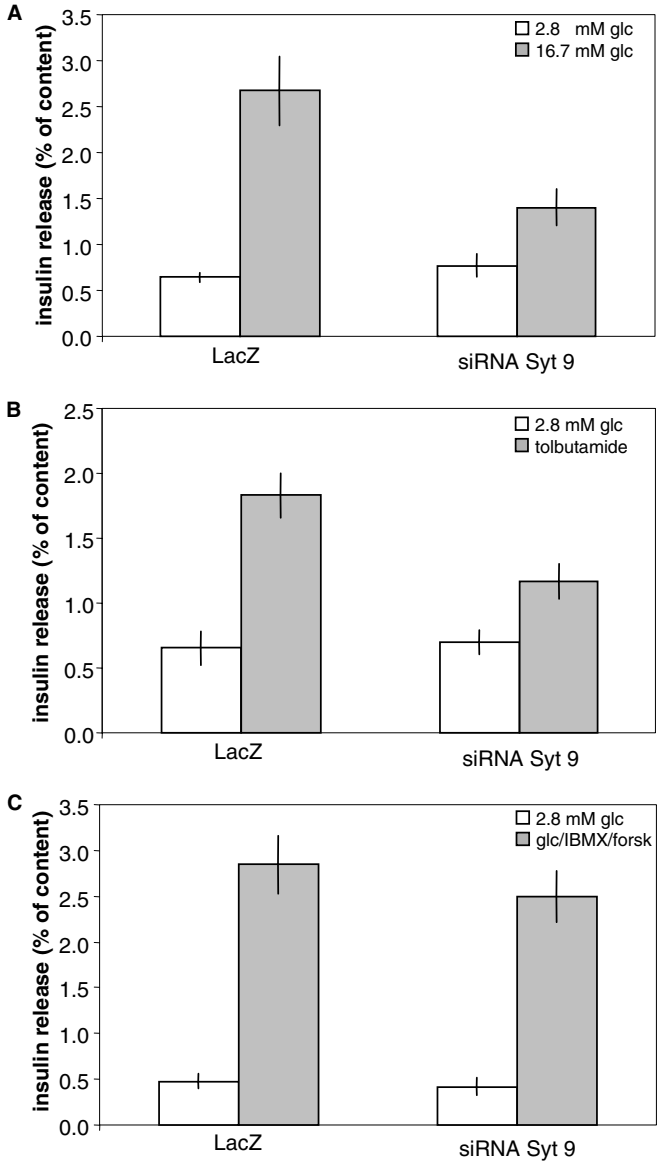

Fig. 4. Effect of Syt 9 silencing on insulin secretion in islets. LacZ or siRNA Syt 9-infected islets were incubated for 30 min at basal 2.8 mM glucose (glc) or stimulated with 16.7 mM glucose (A), with 200 μM tolbutamide (B) or with 16.7 mM glucose, 100 μM IBMX, 1 μM forskolin (C). Data represent the means ± S.E. of 3 independent experiments performed in triplicates.

Syt 9 can interact with phospholipids [22] and SNAP 25 [6] at <10 μM Ca^{2+}, this is again consistent with a functional involvement of Syt 9 in the slow insulin exocytosis.

In conclusion, we have demonstrated that Syt 9 is associated with insulin granules in native β-cells and may function as Ca^{2+} sensor for the slow component of release either alone or through Ca^{2+}-dependent interaction with Syt 7 [29]. Taken together with its expression in chromaffin cells (M.F. unpublished observation) Syt 9 may be the relevant Ca^{2+} sensor for exocytosis in primary endocrine cells.

Acknowledgements: We are grateful to Alessandra Strom for helping with the immunostaining of the pancreatic polypeptide cells. We are indebted to Dale Brighouse, Eve-Julie Sarret, Kristina Borglid and Lina Gefors for technical assistance. This work was supported by the Swiss National Science Foundation Grant (32-66907.01) CBW and the Swedish Medical Research Council Grant (13147).

References

[1] Rorsman, P. and Renstrom, E. (2003) Insulin granule dynamics in pancreatic beta cells. Diabetologia 46, 1029–1045.
[2] Sudhof, T.C. (2002) Synaptotagmins: why so many? J. Biol. Chem. 277, 7629–7632.
[3] Fukuda, M. (2003) Synaptotagmins, Ca^{2+}- and phospholipid-binding proteins that control Ca^{2+}-regulated membrane trafficking. Recent Res. Devel. Chem. Phys. Lipid 1, 15–51.
[4] Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F. and Sudhof, T.C. (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79, 717–727.
[5] Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K. and Kumakura, K. (1997) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells. Proc. Natl. Acad. Sci. USA 94, 287–291.

[6] Zhang, X., Kim-Miller, M.J., Fukuda, M., Kowalchyk, J.A. and Martin, T.F. (2002) Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. Neuron 34, 599–611.
[7] Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K. and Wollheim, C.B. (1997) The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic beta-cells: action of synaptotagmin at low micromolar calcium. EMBO J. 16, 5837–5846.
[8] Fukuda, M. (2004) RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells. Biochem. J. 380, 875–879.
[9] Brown, H., Meister, B., Deeney, J., Corkey, B.E., Yang, S.N., Larsson, O., Rhodes, C.J., Seino, S., Berggren, P.O. and Fried, G. (2000) Synaptotagmin III isoform is compartmentalized in pancreatic β-cells and has a functional role in exocytosis. Diabetes 49, 383–391.
[10] Gao, Z., Reavey-Cantwell, J., Young, R.A., Jegier, P. and Wolf, B.A. (2000) Synaptotagmin III/VII isoforms mediate Ca^{2+}-induced insulin secretion in pancreatic islet β-cells. J. Biol. Chem. 275, 36079–36085.
[11] Gut, A., Kiraly, C.E., Fukuda, M., Mikoshiba, K., Wollheim, C.B. and Lang, J. (2001) Expression and localisation of synaptotagmin isoforms in endocrine β-cells: their function in insulin exocytosis. J. Cell. Sci. 114, 1709–1716.
[12] Iezzi, M., Kouri, G., Fukuda, M. and Wollheim, C.B. (2004) Synaptotagmin V and IX isoforms control Ca^{2+}-dependent insulin exocytosis. J. Cell. Sci. 117, 3119–3127.
[13] Saegusa, C., Fukuda, M. and Mikoshiba, K. (2002) Synaptotagmin V is targeted to dense-core vesicles that undergo calcium-dependent exocytosis in PC12 cells. J. Biol. Chem. 277, 24499–24505.
[14] Rickman, C., Craxton, M., Osborne, S. and Davletov, B. (2004) Comparative analysis of tandem C2 domains from the mammalian synaptotagmin family. Biochem. J. 378, 681–686.
[15] Fukuda, M., Ogata, Y., Saegusa, C., Kanno, E. and Mikoshiba, K. (2002) Alternative splicing isoforms of synaptotagmin VII in the mouse, rat and human. Biochem. J. 365, 173–180.
[16] Merglen, A., Theander, S., Rubi, B., Chaffard, G., Wollheim, C.B. and Maechler, P. (2004) Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 145, 667–678.
[17] Ishihara, H., Maechler, P., Gjinovci, A., Herrera, P.L. and Wollheim, C.B. (2003) Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. Nat. Cell. Biol. 5, 330–335.
[18] Nagelhus, E.A., Veruki, M.L., Torp, R., Haug, F.M., Laake, J.H., Nielsen, S., Agre, P. and Ottersen, O.P. (1998) Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Muller cells and fibrous astrocytes. J. Neurosci. 18, 2506–2519.
[19] Gauthier, B.R., Iezzi, M., Fukuda, M., Duhamel, D.L. and Wollheim, C.B. (2004) The synaptotagmin VII spliced variants β and Δ are expressed in native islets β-cells and regulate insulin exocytosis (Abstract). Diabetologia 47 (Suppl 1), 78A.
[20] Butz, S., Fernandez-Chacon, R., Schmitz, F., Jahn, R. and Südhof, T.C. (1999) The subcellular localizations of atypical synaptotagmins III and VI. Synaptotagmin III is enriched in synapses and synaptic plasma membranes but not in synaptic vesicles. J. Biol. Chem. 274, 18290–18296.
[21] Fukuda, M., Kanno, E. and Mikoshiba, K. (1999) Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. J. Biol. Chem. 274, 31421–31427.
[22] Shin, O.H., Maximov, A., Lim, B.K., Rizo, J. and Sudhof, T.C. (2004) Unexpected Ca^{2+}-binding properties of synaptotagmin 9. Proc. Natl. Acad. Sci. USA 101, 2554–2559.
[23] Fukuda, M., Kowalchyk, J.A., Zhang, X., Martin, T.F. and Mikoshiba, K. (2002) Synaptotagmin IX regulates Ca^{2+}-dependent secretion in PC12 cells. J. Biol. Chem. 277, 4601–4604.
[24] Barg, S., Ma, X., Eliasson, L., Galvanovskis, J., Gopel, S.O., Obermuller, S., Platzer, J., Renstrom, E., Trus, M., Atlas, D., Striessnig, J. and Rorsman, P. (2001) Fast exocytosis with few Ca^{2+} channels in insulin-secreting mouse pancreatic β-cells. Biophys. J. 81, 3308–3323.
[25] Henquin, J.C. (1980) Specificity of divalent cation requirement for insulin release. Effects of strontium. Pflug. Arch. 383, 123–129.
[26] Proks, P. (1995) Ashcroft FM: effects of divalent cations on exocytosis and endocytosis from single mouse pancreatic beta-cells. J. Physiol. 487, 465–477.
[27] Yang, Y. and Gillis, K.D. (2004) A highly Ca^{2+}-sensitive pool of granules is regulated by glucose and protein kinases in insulin-secreting INS-1 cells. J. Gen. Physiol. 124, 641–651.
[28] Wan, Q.F., Dong, Y., Yang, H., Lou, X., Ding, J. and Xu, T. (2004) Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca^{2+}. J. Gen. Physiol. 124, 653–662.
[29] Fukuda, M., Kanno, E., Satoh, M., Saegusa, C. and Yamamoto, A. (2004) Synaptotagmin VII is targeted to dense-core vesicles and regulates their Ca^{2+}-dependent exocytosis in PC12 cells. J. Biol. Chem. 279, 52677–52684.